Fulvic acid supplementation and selenium deficiency disturb the structural integrity of mouse skeletal tissue

An animal model to study the molecular defects of Kashin–Beck disease

Chunlin YANG,*§ Congrong NIU,* Michael BODO,* Elmar GABRIEL,† Holger NOTBOHM,* Eduard WOLF‡ and Peter K. MÜLLER*

* Institute of Medical Molecular Biology, Medical University of Lübeck, 2400 Lübeck, † Surgical Clinic, University of Würzburg, Würzburg, and ‡ Institute of Pathology, University of Hamburg, Hamburg, Germany

High concentrations of fulvic acid and selenium deficiency are the main causative factors of Kashin–Beck disease, an endemic, chronic and degenerative osteoarticular disorder found in China. In the search for an animal model of this disease, mice were exposed to these pathogenetic conditions for two generations and the collagen types from skin, bone and cartilage were analysed. The growth of the treated mice was slightly retarded, and the rate of reproduction was lower in animals maintained on a fulvic acid-supplemented and/or selenium-deficient diet. Irregular bone formation was seen by radiography and morphometry. Biochemical analysis indicated that lysine residues in collagen I from bone and in collagen II from cartilage were overmodified. The values of Hyl/(Hyl+Lys) in bone collagen α1(I) chains from treated mice were about 0.434–0.484, i.e. substantially higher than that of the control (0.277). The values of this parameter for collagen II were 0.482 for control and 0.546–0.566 for treated mice. The melting temperature of collagen I from bones of treated mice was 1 °C lower than that of control collagen, indicating decreased thermal stability. The breakage point of the tibiae of treated mice occurred at a lower preload force than for controls, suggesting that the overmodified and thermally less stable collagen molecules are causally related to a lower mechanical strength of bones.

INTRODUCTION

Selenium is a nutritional trace element which constitutes a part of the active sites of glutathione peroxidase and type I iodothyronine deiodinase [1,2]. Both of these enzymes play crucial roles in the organism: glutathione peroxidase can protect tissues against reactive oxygen damage, and iodothyronine deiodinase can convert thyroxine into the biologically active form 3,5,3′-tri-iodothyronine. Fulvic acid, which occurs naturally in water, soil and peat as a humic fraction is soluble in water and in aqueous acidified solution. It is produced by chemical and microbial decomposition of plant and animal material and contains many organic compounds with reactive groups, e.g. carboxyls, hydroxyls, carbonyls, phenols and quinones [3,4]. Kashin–Beck disease (KBD), which occurs mainly in China, is an endemic, degenerative, osteoarticular disorder associated with severe skeletal deformation and dwarfism. The epidemiology of KBD provides convincing evidence that selenium deficiency in the diet and a high content of fulvic acid in the drinking water are the main causative factors of the disease in China [5,6]. Dietary supplementation of selenium to populations in endemic areas of China and an improvement in the quality of the drinking water have substantially reduced the number of new cases of KBD [7]. Little is known about affected animals in the endemic areas of China, and no animal model to study the disease is currently available.

Multiple degenerative and necrotic lesions within the articular cartilage and the growth plate represent the initial pathological changes which are associated with disturbed mineralization and disfiguration of joints [8]. Furthermore, morphological studies on affected tissues show a distorted deposition of collagen fibres in cartilage. Recently we described a molecular defect in collagen II from the articular cartilage of two KBD patients. Specifically, we showed overmodification and impaired conversion of pN-collagen II to collagen II [9]. Since collagens are the main structural proteins of the extracellular matrix of bone and cartilage, they play a crucial role in the maintenance of the biological function of skeletal tissues. This notion is supported by studies of inherited diseases of connective tissue, which have demonstrated that mutations in collagen genes as well as defects in the post-translational processing of collagens may result in pathological conditions [10–12]. KBD, in contrast, is an acquired rather than an inherited disease. Studies on the relationship between the proposed causative factors of the disease and collagen metabolism, as well as on the structure of joints, may contribute to the understanding of both the molecular basis and the aetiology of KBD.

In the present work, we aimed to clarify the impact of selenium deficiency and of fulvic acid supplementation on the development of the skeletal system and on the post-translational modification of collagen in these tissues. Mice were fed on a selenium-deficient diet and/or had fulvic acid added to their drinking water for two generations. The growth rate, the ability to reproduce and the morphology of the knee joints of the treated mice were compared with those of control animals. The collagen composition and the degree of collagen modification were studied in tissue extracts of skin and joints. The thermal stability of individual collagen molecules and the mechanical strength of the tibia were analysed by biophysical methods.

MATERIALS AND METHODS

Isolation of fulvic acid

Drinking water from a KBD-affected area was acidified with HCl and subsequently passed over a GDX-102 resin (Tianjin 2nd Chemical Factory, China). The water-soluble fulvic acid was absorbed on the resin and eluted with a solvent containing ethanol/ammonia (1:2, v/v). From 1000 litres of drinking water,

Abbreviation used: KBD, Kashin–Beck disease.

§ To whom correspondence and reprint requests should be addressed.

about 300 mg of fulvic acid was collected and stored at 4 °C until use [6].

Animal care and feeding

Four groups of NMRi mice were supplied with food and drinking water as follows: (1) control group, with standard diet; (2) FA group, with standard diet and fulvic acid-supplemented drinking water; (3) Se-D group, with selenium-deficient diet; and (4) Se-D + FA group, with both a selenium-deficient diet and fulvic acid-supplemented drinking water. The fulvic acid-supplemented drinking water contained 211 p.p.m. fulvic acid. The selenium-deficient diet (C 1045 Selen-arm, Altromin, Germany) had a concentration of 31 p.p.b. selenium, whereas the selenium content of standard feed is 314 p.p.b.

Two male and seven female mice in each group (50 days old) were given their individual diets and water for 2 weeks. The male and female animals were then put together for 1 week. Thereafter, the female mice were separated and fed individually. New-born mice (second generation) were maintained on the same individual diets as their parents, and 10 second-generation mice from each group (21 days old) were killed for preparation of collagen. Other mice from each group were fed on the diets up to 49 days of age, in order to investigate the bone formation and mechanical strength of intact tibia.

Isolation of collagen types from the knee joints and the skin of mice

All procedures from the extraction and separation of collagen were performed at below 4 °C. The skin and the entire knee joints from 10 animals (21 days old) were prepared and pooled. The knee joints consisted of both cartilaginous and bony parts. After homogenization with a stainless steel ball-mill under liquid nitrogen, each sample was washed with PBS and 96 % ethanol to remove residual fat and blood. The tissue powder was extracted with 1 M NaCl and 0.05 M Tris, pH 7.4, for 2 days in the presence of 5 mM phenylmethanesulphonyl-fluoride and 10 mM EDTA as proteinase inhibitors. The insoluble residues were extracted with 0.1 mg/ml pepsin in 0.2 M sodium acetate, pH 1.5, for 24 h. After centrifugation, the supernatant was neutralized with saturated Tris solution to inactivate pepsin. The pepsin extraction was repeated five times, and all neutralized tissue extracts were pooled for selective separation of individual collagen types.

The pepsin extracts of skin were first equilibrated in 0.5 M NaCl and 0.05 M Tris, pH 7.4, and subsequently dialysed against 1.8 M NaCl and 0.05 M Tris, pH 7.4, to separate collagen III. Collagen I was precipitated by a further dialysis step against 2.5 M NaCl and 0.05 M Tris, pH 7.4. The precipitate containing collagen I was collected by centrifugation, while collagen V remained in the supernatant. The pepsin extract joints was dialysed against 0.9 M NaCl and 0.5 M acetic acid. After centrifugation, the supernatant was dialysed against 1.2 M NaCl/0.5 M acetic acid and then against 2.0 M NaCl/0.5 M acetic acid to precipitate collagens IX and XI [13]. The pellets were dissolved in 0.5 M NaCl and 0.05 M Tris, pH 7.4, for preparation of collagens I, II and III. Collagen III was precipitated by dialysis against 1.8 M NaCl and 0.05 M Tris, pH 7.4, and collected by centrifugation. Collagen I was precipitated in 2.5 M NaCl and 0.05 M Tris, pH 7.4. The remainder was first dialysed against 0.5 M NaCl and 0.05 M Tris, pH 7.4, and then against 0.7 M NaCl and 0.5 M acetic acid to precipitate collagen II. All preparations of collagen were extensively dialysed against 0.05 % acetic acid and stored at −20 °C in the same solvent. The identity of each collagen type was checked by SDS/PAGE.

SDS/PAGE analysis

Aliquots of each sample were lyophilized and dissolved in SDS sample buffer at a concentration of about 1 mg/ml. Stacking polyacrylamide gels (4 %) and separation gels (6 %) were used to separate collagen chains, and delayed reduction was performed for the identification of collagen III [14]. Gels were stained with Coomassie Blue and destained with dilute acetic acid under gentle shaking.

Immunoblotting

Following the electrophoretic separation of the neutral salt extract of the knee joints, protein bands were blotted on to a nitrocellulose filter by semi-dry electrotransfer and probed using an antiserum against mouse collagen II. The protein bands were visualized by probing with alkaline phosphatase-conjugated second antibodies as described previously [9].

Preparation of antisera against collagen

Antisera against mouse collagen II were raised in rabbits. The antibody titre was determined by a direct e.l.i.s.a. and the specificity of the antiserum was checked by immunohistological staining of suitable tissue sections and by immunoblotting [15].

Morphological observations

Skin, muscles and tendons were removed from the knee joints of the mice and the hard tissues of the joints were fixed and embedded in methyl methacrylate without decalcification. The phosphate in the prepared section was visualized by von Kossa staining [16].

Isolation of α1(I), α2(I) and α1(II) collagen chains by h.p.l.c.

Pepsin-solubilized and salt-fractionated pools of collagen I were chromatographed first on a molecular sieve column (Superose TM6, Pharmacia, Freiburg, Germany). The column was eluted with 0.15 M NaCl, 0.05 M NaH_2PO_4 and 4 M urea, pH 6.5, at a flow rate of 0.1 ml/min to remove higher-molecular-mass components of collagen. The fraction containing monomers was directly loaded on a Waters C_{18} reverse-phase column. The separation of α1(I) from α2(I) chains was performed with a linear gradient of 21–32 % (w/v) acetonitrile in water with 0.1 % (w/v) trifluoroacetic acid for 37 min at a flow rate of 1 ml/min. Individual fractions were analysed by SDS/PAGE. α1(II) chains of cartilage collagen were purified by the same procedure [17].

Amino acid analysis and glycosides of hydroxylysine

The amino acid composition of lyophilized samples was determined after hydrolysis with 6 M HCl (Pierce) at 110 °C under nitrogen for 22 h on a Beckman 6300 amino acid analyser (Beckman, München, Germany) equipped with a 250 mm × 3.4 mm column. A mixture of amino acids with a composition similar to that of a collagen hydrolysate was prepared from commercially available standards (Aldrich, Steinheim, Germany) and used for calibration [18].

Hydroxylysine glycosides and hydroxylysine were purified from alkaline hydrolysates (2 M KOH, 24 h, 110 °C, in 2 ml polypropylene reaction vials) of the lyophilized sample by cation-

exchange chromatography on Dowex 50W-X8, as described by Tenni et al. [19]. Eluates were lyophilized, dissolved in sample buffer (Na-S; Beckman) for amino acid analysis and separated on the amino acid analyser as described above with a step gradient composed of 7.3 ml of buffer Na-F and 3.2 ml of buffer Na-D (Beckman) at a column temperature of 77 °C and a flow rate of 20 ml/h. A glucosyl-galactosyl-hydroxylysine standard was prepared according to Tenni et al. [19] and used for quantification of glucosyl-galactosyl-hydroxylysine and galactosyl-hydroxylysine [19].

C.d. and transition profiles

C.d. spectra were recorded on a Jasco J-500 A spectropolarimeter, equipped with a temperature-controlled Gilford quartz cell of 1 cm path-length. The molar ellipiticity was calculated on the basis of a mean residue mass of 98 g/mol. Zero helix-coil conversion corresponded to the ellipticity of the sample measured at 20 °C, and 100 % conversion corresponded to the ellipticity at 50 °C and 221 nm, typical for totally denatured collagen. Thermal transition curves were recorded at a fixed wavelength (221 nm) by raising the temperature linearly at a rate of 30 °C/h using a Gilford temperature programmer. The sample was present at about 10 μg/ml in 0.05 % acetic acid. A change in the thermal stability of collagen due to radiation damage could not be detected [20]. The accuracy of the measurements was $\pm$0.1 °C.

Resistance to proteolysis [21]

Collagen samples were dissolved in 0.4 M NaCl and 0.1 M Tris, pH 7.4, to a final concentration of 2 mg/ml. Aliquots of 50 μl were continually warmed from 33 °C up to 43 °C at a rate of 12 °C/h using a temperature programmer. At certain intervals aliquots were withdrawn and immediately quenched to 20 °C; 10 μl of trypsin (1 mg/ml) was added. After digestion for 2 min, 50 μg of soybean-extracted trypsin inhibitor was added in 10 μl of water. Degradation products were analysed by SDS/PAGE.

Dynamic mechanical behaviour and breakage strength of bone [22]

Five hind-leg tibiae from each group were carefully freed from adhesive muscle, tendon and other soft tissue. The ends (condyles of joints) were fixed with Epoxide casting on thin metal plates (10 mm × 10 mm) to provide a defined support for three-point bending tests (see Fig. 4 inset). The test was performed using a Dynamic Mechanical Spectrometer Eplexor (Fa. Gabo-Qualimeter GmbH, D-3031 Ahlden, Germany). The bones were loaded stepwise with increased static preload forces (step-width 0.5 N) and with superimposed dynamic forces at 1 Hz (step-width 0.25 N, up to 2 N maximum load), until the breakage point was reached. The elastic component E' and the viscous component E'' of the complex Young modules E^* were determined as the corresponding spring-constant values in N/mm. The elastic moduli (in N/mm^2) cannot be calculated, as the geometry with the area momentum of inertia is not known and could not be determined.

RESULTS

Growth and reproduction of mice

Selenium is an essential trace element for humans and animals. Selenium deficiency in animals can be established by suitable diets that are either deficient in selenium or supplemented with compounds which interfere with selenium utilization [23]. In the experiments described here, mice were fed on a commercially available selenium-deficient diet containing about 10 % of the selenium concentration usually present in standard mouse diet (31 p.p.b. versus 314 p.p.b. of selenium). Water may contain significant amounts of selenium depending on the location [22], and thus deionized water was supplied to the mice. Mice were given the selenium-deficient diet and/or fulvic acid-containing drinking water for two generations. The first generation of selenium-deficient mice already showed a deleterious impact with regard to number of pregnancies and to the survival rate of newborn mice. Furthermore, the growth of the second-generation mice was retarded. Similar results were reported with rats maintained on a selenium-deficient diet [23].

Fulvic acid is a chemically ill-defined mixture of organic compounds whose composition and chemical properties may vary depending on its origin [24]. Here we used fulvic acid extracted from drinking water collected in a KBD-affected area of China. There was no significant influence of fulvic acid supplementation on the growth of mice, although their reproduction was markedly affected. A combination of the selenium-deficient diet along with fulvic acid-supplemented drinking water did not result in a cumulative impact.

Morphological observations

Radiographs of the epiphyses of mice showed differences between the control and treated mice. A thin and rough calcification

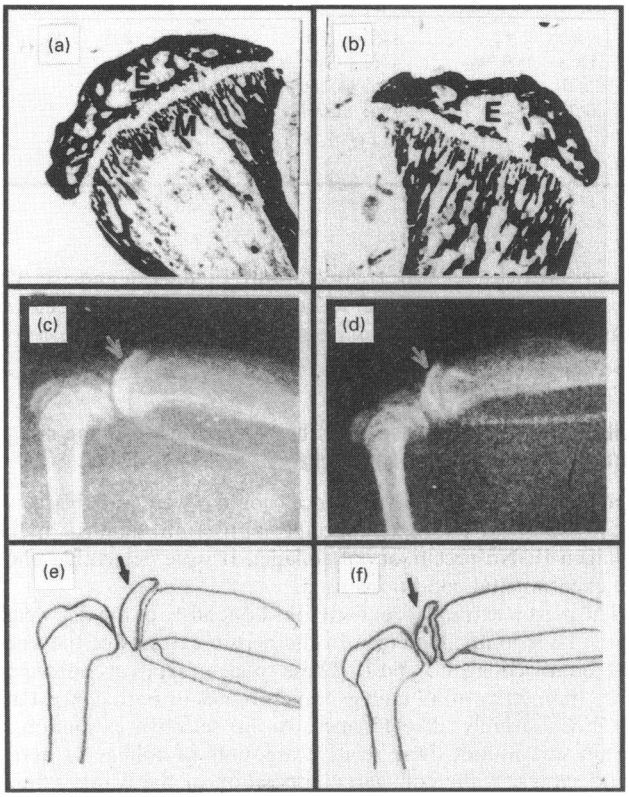

Figure 1 Bone formation in control mice (a, c, e) and in mice after treatment with fulvic acid-containing water and a selenium-deficient diet (b, d, f)

(**a, b**) Distribution of phosphate in tibial epiphysis (E). An irregular shape of calcified tissue in the epiphysis of the treated mice can be observed. Also shown are radiographs (**c, d**) and schematic diagrams (**e, f**) of the hind legs of mice. The tibial epiphysis (arrows) of treated mice shows a thin and rough calcified ring in place of a smooth and homogeneous absorption profile.

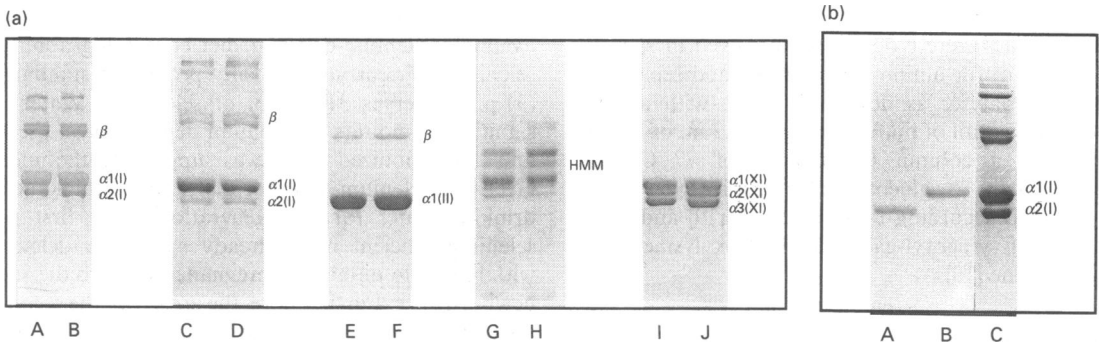

Figure 2 Analysis of collagen types by SDS/PAGE

(**a**) Collagen types in pepsin extracts of the knee joints of control mice (lanes A, C, E, G and I) and mice treated with fulvic acid-containing water and a selenium-deficient diet (lanes B, D, F, H and J). Lanes A and B show unseparated extracts. Collagen types were differentially separated by salt precipitation into collagen I (C and D), collagen II (E and F), then high-molecular mass (HMM) component of collagen IX (G and H) and collagen XI (I and J). The peptides were separated on a 6% polyacrylamide gel and stained by Coomassie Blue. (**b**) α1(I) (chains (lane B) and α2(I) chains (lane A) of collagen I (lane C) were purified by f.p.l.c. on a TM6 molecular-sieve column and further separated by h.p.l.c. on a reverse-phase C_{18} resin. The peptides were separated by SDS/PAGE and stained as above.

Table 1 Hydroxylation of lysine and proline residues in collagen I and collagen II from mice

The values for pooled material from 10 mice in each group are expressed as means ± S.D. Statistical analysis was done using an ANOVA program (SPSS Inc., Chicago, U.S.A.) based on one-way analysis of variance.

	α1(I) of bone collagen I		α1(I) of skin collagen I		α1(II) of collagen II	
Group	Hyp/(Hyp + Pro)	Hyl/(Hyl + Lys)	Hyp/(Hyp + Pro)	Hyl/(Hyl + Lys)	Hyp/(Hyp + Pro)	Hyl/(Hyl + Lys)
Control	0.456 ± 0.006	0.277 ± 0.005	0.426 ± 0.011	0.145 ± 0.007	0.465 ± 0.015	0.482 ± 0.016
FA	0.467 ± 0.005	0.434 ± 0.005	0.438 ± 0.006	0.161 ± 0.006	0.466 ± 0.007	0.552 ± 0.11
Se-D	0.452 ± 0.005	0.484 ± 0.012	0.446 ± 0.006	0.152 ± 0.004	0.476 ± 0.005	0.566 ± 0.009
Se-D + FA	0.458 ± 0.004	0.465 ± 0.013	0.432 ± 0.013	0.142 ± 0.008	0.470 ± 0.024	0.546 ± 0.021
P	> 0.05	< 0.01	> 0.05	> 0.05	> 0.05	< 0.01

pattern was visible in the treated group (Figures 1a and 1b). Von Kossa staining of epiphyseal sections of mouse tibiae also showed that the treated mice had an irregularly shaped calcification zone (Figure 1).

Collagen types in the knee joints of mice, and preparation of α1(I), α2(I) and α1(II) collagen chains

Neutral salt extracts of mice joints were analysed by immunoblotting using a specific antiserum against mouse collagen II. No precursors of collagen II were detectable under the experimental conditions used.

The pepsin extract accounted for about 80% of the dry weight of the knee joints. Collagen in the pepsin extracts of the knees was mainly collagen I and II. These collagen types accounted for more than 90% of all collagenous proteins in both control and the most severely affected mice. As no selective extraction by pepsin was found, these relative amounts of collagen I and II could represent the collagen composition of the whole sample. Minor collagens, such as collagens IX, XI and III, were collected by differential salt precipitation. No differences in the electrophoretic mobilities or relative amounts of various collagen types could be observed between the control mice and any of the treated groups (Figure 2a).

To obtain pure α1(I) and α2(I) collagen chains and to avoid the disturbance of cross-linking in the amino acid analysis, the β and γ components were removed by f.p.l.c. using a molecular sieve column. Pure α1(I) and α2(I) chains were obtained by reverse-phase h.p.l.c. and used for amino acid analysis. SDS/PAGE of collagen I and of purified α1(I) and α2(I) chains from the control group is shown in Figure 2(b). α1(II) collagen chains were also chromatographically purified and checked by SDS/PAGE and immunoblotting (results not shown).

Hydroxylation and glycosylation

Amino acid analysis was carried out for α1(I) chains of collagen I from bone and skin and for α1(II) chains of collagen II, and the proportions of the post-translationally modified amino acids hydroxyproline and hydroxylysine were calculated. No impact was seen on the hydroxylation of proline and lysine residues of collagen I from skin. However, both the α(I) and α2(II) chains of collagen I isolated from the bones of mice in the FA, Se-D and Se-D + FA groups showed remarkable overhydroxylation of lysine residues, although proline hydroxylation was not altered (Table 1). Selenium deficiency alone caused a similar stimulation of lysine hydroxylation to that observed in the Se-D + FA group. Proline residues in cartilage collagen II were virtually identical among the groups studied. Lysine hydroxylation, however, was similarly elevated in all treated groups (Table 1), although to a minor extent compared with the effects on collagen I. It is known

Table 2 Glycosylation of hydoxylysine residues in collagen II from mice

The values for pooled material from 10 animals in each group are presented as means ± S.D. GGH, α-D-glucosyl-β-D-galactosyl-hydroxylysine; GH, α-D-galactopyranosyl-hydroxylysine. Statistical analysis on the significance of variance was done using an ANOVA program (SPSS Inc., Chicago, U.S.A.) based on one-way analysis of variance.

	Glycosylation (residues/1000 amino acid residues)		
Group	GGH	GH	Hyl
Control	3.2 ± 0.2	5.6 ± 0.1	7.5 ± 0.15
FA	4.4 ± 0.1	4.7 ± 0.4	9.0 ± 0.2
Se-D	5.0 ± 0.2	6.6 ± 0.1	8.4 ± 0.1
SE-D + FA	4.6 ± 0.1	6.5 ± 0.1	8.2 ± 0.1
P	< 0.01	< 0.05	< 0.01

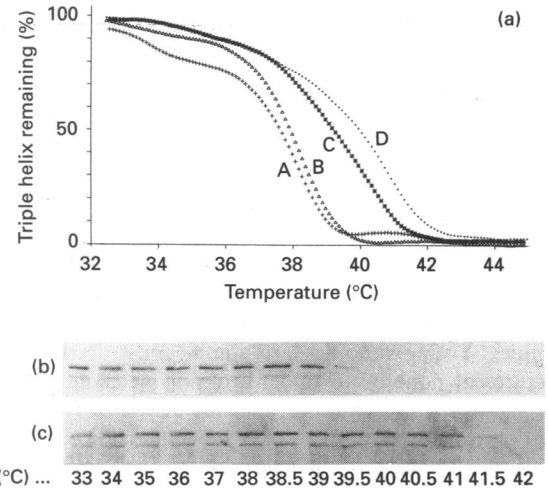

Figure 3 Thermal stability of collagen I

(**a**) The melting curves are shown of collagen I from skin (A and B) and bone (C and D) of control mice (A and D) and of mice treated with fulvic acid-containing drinking water and a selenium-deficient diet (B and C). The melting curves were obtained by measuring the relative molar elipticity at a fixed wavelength (221 nm) detected by c.d. on raising the temperature at a rate of 30 °C/h. (**b**, **c**) SDS/PAGE analysis shows the thermal stability of the triple helix of collagen I from bone of control (**c**) and treated (**b**) mice, as monitored by the resistance to digestion with trypsin on raising the temperature from 33 °C to 43 °C.

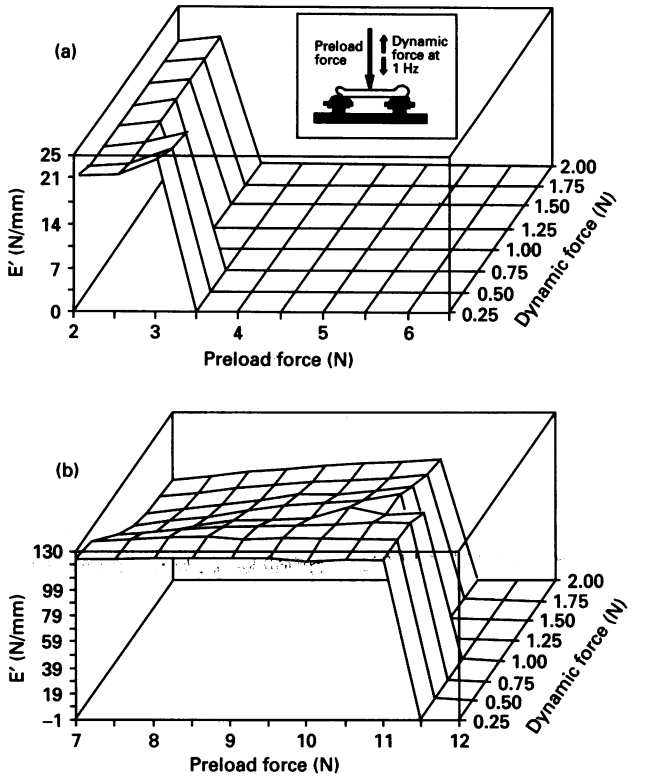

Figure 4 Load-dependent elastic behaviour and breakage points of mice tibiae

(**b**) Tibiae from the control group showed a high resistance against bending and a high static preload force (11.5 N) at the breakage point. (**a**) Tibiae from the affected group showed a low resistance against bending and a low static preload force (3 N) at the breakage point. The inset shows the bending test procedure by dynamic mechanical spectroscopy at an increased loading rate, which results in the destructive breakage of the bone at the breakage point.

that collagen II has a higher basal level of lysine hydroxylation than collagen I [25].

The analysis of di- and mono-glycosides attached to the hydroxylysine residues of collagen II showed only a small increase of α-D-glucopyranosyl-β-D-galactopyranosyl-hydroxylysine and α-D-galactopyranosyl-hydroxylysine in treated groups (Table 2). Further experiments will be necessary to obtain a more thorough analysis and to show a dependence of lysine glycosylation, if any, on the causative factors of KBD.

Thermal stability and resistance of collagen to proteolytic digestion

It is possible that overhydroxylation of lysine in collagens from bone and cartilage could influence some physical properties of the collagen molecules that are crucial for *in vivo* functions. Thus we analysed the thermal stability of collagen II from cartilage and of collagen I from bone and skin by c.d. (Figure 3). Collagen I from the skin of mice in every group showed a near-identical melting temperature (38.0 °C). However, collagen I from the bones of the control mice had a melting temperature of 40.9 °C, which is 1 °C higher than that of collagen I from the bones of the animals under dietary influence. Similarly, collagen II from the cartilage of the control group had a melting temperature of 38.7 °C, which is about 0.4 °C higher than that of collagen II from the treated groups (37.3 °C).

Collagen I molecules from the bones of both the control and the Se-D + FA groups were tested for their stability to proteolytic attack by trypsin over a temperature range of 33–43 °C (Figure 3). SDS/PAGE showed that collagen I from the bones of the treated group was almost completely degraded at 40.5 °C, whereas collagen I from the bones of the control group was stable at that temperature, and was degraded only at 41.5 °C. Thus limited proteolysis and c.d. measurement give similar results with regard to the triple-helical stability of collagen molecules.

Mechanical strength of mouse bones

An important functional property of bone is its ability to bear loads and to provide the skeletal scaffold for the body. The structural integrity of the tissue is a prerequisite for appropriate functioning. Interestingly, measurement of the viscoelastic

properties of the tibiae of the mice showed that the preload force at the breakage point for control mice was in the range 8.5–11.5 N, which was much higher than that of mice subjected to fulvic acid supplementation and selenium deficiency (2.75–5 N). A representative sample of each group is shown in Figures 4(a) and 4(b). This shows that the tibiae of control mice have greater mechanical strength and can bear a larger load than the tibiae of mice maintained on a selenium-deficient and fulvic acid-supplemented diet.

DISCUSSION

The study of the molecular basis of KBD is greatly hampered by the limited access to patients living in rural areas of China and by the virtual lack of affected tissue for biochemical investigation. Although it is not known whether animals living in KBD endemic regions show pathological manifestations typical of KBD, an animal model of the disease would greatly facilitate the molecular analysis of the underlying defects. In the present study we found that selenium-deficient mice and fulvic acid-supplemented mice showed a lower rate of reproduction than controls, and the selenium-deficient mice also showed signs of growth retardation as judged by the decreased body weight. The combination of both a selenium-deficient diet together with fulvic acid supplementation had no cumulative effect on any physiological or biological parameter analysed.

Although no gross skeletal alteration was seen, histological examination of tissue sections showed an irregular mineralization of the tibial epiphysis of the treated mice. A rough and thin X-ray absorption pattern similar to this has been observed with the finger joints of KBD patients [26].

Compositional analysis of collagen types in the knee joints did not reveal any differences between the groups of mice. Amino acid analysis, however, revealed significant overhydroxylation of lysine residues in collagen I from bone of treated animals. Interestingly, the altered modification was tissue-specific, since collagen I from the skin of the same mice was not affected. Collagen II from the cartilage of treated mice was slightly lysine-overhydroxylated and was also overglycosylated. In all instances, hydroxylation of proline was not affected.

Hydroxylation of lysine and proline residues and the glycosylation of some hydroxylysine residues are intracellular events which take place on the nascent collagen chains [27]. Once the triple helix has been formed, no further hydroxylation or glycosylation occurs. Changes in the degree of lysine hydroxylation are seen during embryonic development and tissue repair, and in osteogenesis imperfecta and osteoporosis, two clinical syndromes which are characterized by fragile bones [28,29]. There is also recent evidence that minor variations in proline hydroxylation may occur, depending on the tissue from which the collagen was isolated [30]. Experimental evidence also suggests that the enzymes responsible for lysine or proline hydroxylation would respond differently to changing environmental conditions [31]. Although much is known about the enzymes and the cofactors of collagen hydroxylation and glycosylation, little is known about the factors and conditions which modulate collagen hydroxylation in response to physiological demands and tissue repair.

Since selenium is a part of the active site of the enzyme glutathione peroxidase, which could act as a scavenger of reactive oxygen, deficiency of this trace element may lead to excess of free radicals. Fulvic acid from the drinking water of KBD affected areas has a high concentration of semiquinone radicals, which can generate superoxide [6]. We assume that either selenium deficiency or fulvic acid supplementation used in these experiments may alone induce a maximal level of free radicals, which is not further stimulated by addition of the other treatment.

Fe^{2+}, ascorbate(Vc) and molecular oxygen are essential for collagen hydroxylation, and constitute a typical system for the production of reactive oxygen species [32,33]. There is experimental evidence indicating that activated oxygen is bound to Fe^{2+} and is involved in the collagen hydroxylation in the form of superoxide [34]. It is conceivable that these enzyme–cofactor complexes are different for proline and lysine hydroxylation, and may also respond differently to the changing environmental conditions. It is also conceivable that the rather high degree of proline hydroxylation achieved under normal physiological conditions cannot be stimulated further because the remaining proline residues cannot be used as substrates. Interestingly, overmodified collagen I was found in Dupuytren's contracture, where it was associated with increases in the concentration of hypoxanthine and in the activity of xanthine oxidase, which again can produce superoxide ([35]; H. Notbohm, unpublished work). Thus we assume that an excess of superoxide can bring about further collagen hydroxylation which is limited only by the structural requirements of the substrate.

It has been reported that overmodification of lysine residues could disturb the thermal stability of collagen molecules as well as altering the properties of collagen fibrils, such as thermal stability, dynamics of fibrollogenesis and size [30,36]. Measurement of thermal stability by c.d. and proteolytic digestion showed that the overmodification of lysine residues is associated with less stable collagen I and II molecules. We also show for the first time that the biomechanical strength of bone containing overhydroxylated collagen is much lower than that of control bones. Both the preload force and the elastic component (E') of complex Young modules are significantly decreased, so that fractures of bone occur under a minor mechanical impact. We favour at present the notion that the action of free radicals has a major impact by stimulating lysine modification, which in turn seems to influence the thermal stability of the collagen molecules and the mechanical strength of the bones. It is, however, conceivable that free radicals may damage other molecules of the extracellular matrix and/or have a specific effect on the metabolic capacities of osteoblasts.

We thank Mrs. I. Fleischauer, C. Sesselmenn and C. Rau for their excellent technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft (Mu 378/16-1) and by a grant from the Wilhelm-Sander-Stiftung (89.035.1).

REFERENCES

1 Leifelder, W., Zehelein, E., Mandrand-Berthelot, M. A. and Bock, A. (1988) Nature (London) **331**, 723–725
2 Behne, D., Kyriakopoulos, A., Meihold, H. and Löhrle, J. (1990) Biochem. Biophys. Res. Commun. **173**, 1143–1149
3 Schnitzer, M. (1978) in Soil Organic Matter (Schnitzer, M. and Khan, S. U., eds.), vol. 8, p. 57, Elsevier, Amsterdam
4 Choudhry, G. G. (1981) Toxicol. Environ. Chem. **4**, 209
5 Jiang, Y. F. and Xu, G. L. (1989) in Selenium in Biology and Medicine (Wendel, A., ed.), pp. 263–269, Springer-Verlag, Berlin
6 Peng, A., Yang, C. L., He, R. G. and Wang, D. H. (1988) Environ. Chem. **7**, 10–15
7 Li, C. Z. (1979) Zhonghua Yixue Zazhi **59**, 169–171
8 Sokoloff, L. (1985) Clin. Rheum. Dis. **11**, 187–202
9 Yang, C. L., Bodo, M., Notbohm. H. and Müller, P. K. (1991) Eur. J. Biochem. **202**, 1141–1146
10 Weil, D., Bernard, M., Combates, N., Wirtz, M. K., Hollister, D. W., Steinmann, B. and Ramirez, F. (1988) J. Biol. Chem. **263**, 8561–8564
11 Lapiér, C. M., Lanaers, A. and Kohn, L. D. (1971) Proc. Natl. Acad. Sci. U.S.A. **68**, 3054–3058
12 Pinnell, S. R., Krane, S. M., Kenzora, J. E. and Glimcher, M. J. (1972) N. Engl. J. Med. **286**, 2594–2598

13 Reese, C. A. and Mayne, R. (1981) Biochemistry **20**, 5443–5448
14 Clark, C. C. and Richard, C. F. (1985) Collagen Relat. Res. **5**, 205–223
15 Timpl, R. (1984) in Extracellular Matrix Biochemistry (Piez, K. A. and Reddi, A. H., eds.), pp. 159–190, Elsevier, New York
16 Bullough, P. G. and Vigorita, V. J. (eds.) (1987) in Orthopädische Krankheitsbilder, pp. 28–34, Thieme, Stuttgart
17 Bateman, J. F., Mascara, T., Chan, D. and Cole, W. G. (1986) Anal. Biochem. **154**, 338–344
18 Bätge, B., Nerlich, A., Brenner, R., Yang, C. L. and Müller, P. K. (1992) Acta Pediatr. **81**, 156–162
19 Tenni, R., Rimodi, D., Zanaboni, G., Cetta, G. and Castellani, A. A. (1984) Ital. J. Biochem. **33**, 117–127
20 Peltonen, L., Palotie, A., Hayashi, T. and Prockop, D. (1980) Proc. Natl. Acad. Sci. U.S.A. **77**, 162–166
21 Bruckner, P. and Eikenberry, E. F. (1984) Eur. J. Biochem. **140**, 397–399
22 Gabiel, E., Bauer, V., Wollnik, Ch. and Gaddum, F. (1989) in Verbundstoffe Stoffverbunde in Technik and Medicin (Ondracek, G., ed.), vol. 2, pp. 79–88, Verlag Deutsche Gesellschaft für Metallkande, Oberursel
23 Burk, R. F. (1987) Methods Enzymol. **143**, 307–313
24 Schnitzer, M. and Levesque, M. (1979) Soil Sci. **127**, 140–145
25 Miller, E. J. (1984) in Extracellular Matrix Biochemistry (Piez, K. A. and Reddi, A. H., eds.), pp. 41–81, Elsevier, New York
26 Mo, D., Wang, Y., Zhu, C., Reng, H., Xing, S., Liu, Y. and Ying, M. (1985) Zhonghua Fangshexue Zazhi **19**, 87–92
27 Kivirikko, K. I. and Myllyia, R. (1980) in The Enzymology of Post-Translational Modifications of Protein (Freedman, R. B. and Hawkins, H. C., eds.), vol. 1, pp. 53–104, Academic Press, London
28 Brenner, R. E., Lehmann, H., Notbohm, H., Nerlich, A., Vetter, U. and Müller, P. K. (1990) Ann. N.Y. Acad. Sci. **580**, 549–551
29 Quinn, R. S. and Krane, S. M. (1979) Biochim. Biophys. Acta **585**, 589–598
30 Notbohm, H., Mosler, S., Bodo, M., Yang, C. L., Lehmann, H., Bätge, B. and Müller, P. K. (1992) J. Protein Chem. **11**, 635–643
31 Torreblanco, A., Adachi, E., Hojima, Y., Wootton, J. A. M., Minor, R. R., and Prockop, D. J. (1992) J. Biol. Chem. **267**, 2650–2655
32 Nerlich, A., Pöschl, E., Voss, T. and Müller, P. K. (1986) Rheumatology **10**, 70–90
33 Halliwell, B. and Gutteridge, J. M. C., eds. (1989) Free Radicals in Biology and Medicine, pp. 166–168, Clarendon Press, Oxford
34 Halliwell, B. and Gutteridge, J. M. C., eds. (1989) Free Radicals in Biology and Medicine, pp. 371–372, Clarendon Press, Oxford
35 Marrell, C., Francis, M. and Bromley, L. (1987) Br. Med. J. **295**, 1373–1375
36 Torre-Blanco, A., Adachi, E., Hojima, Y., Wootton, M., Minor, R. and Prokop, D. (1992) J. Biol. Chem. **267**, 2650–2655

Received 27 March 1992/7 August 1992; accepted 21 August 1992